

THE EFFECT OF LEUCOGENENOL ON
CLASSICAL CHEMOTHERAPY AND IMMUNOTHERAPY
OF FRIEND VIRUS DISEASE

An abstract of a Thesis by
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The problem. These experiments determined the effects of the immunostimulants leucogenenol and Bacille Calmette-Guerin (BCG) in combination with Methotrexate chemotherapy on Friend Virus Disease.

Procedure. FV infected mice were treated with various combinations of the chemo- and immunotherapeutic agents being investigated. Three parameters of the disease, survival time, ratios of spleen and liver weight to total body weight and periodic differential counts of peripheral blood were studied and analyzed.

Findings. Although Methotrexate significantly prolonged survival time in animals thus treated, the other parameters examined were not significantly different from the untreated controls at the time of death. Immunostimulation with leucogenenol, BCG, or both had no appreciable effect on any of the parameters of the disease.

Conclusion. It was concluded that the effect of MTX therapy was to suppress the production and sensitization of T-lymphocytes so efficiently that consequent attempts at restimulation were ineffective. Furthermore, this suppression was sufficiently great so that upon cessation of MTX therapy the proliferation of leukemic cells probably exceeded that of new, immunocompetent T-lymphocytes.

Recommendations. More studies on the molecular and cellular action of leucogenenol are necessary. An important question raised by this study is which kind of lymphocytes are stimulated by leucogenenol. If only B-lymphocytes are affected, the overall result may be to enhance tumor growth by a blocking effect on the tumor receptor sites. Further, leucogenenol should be tried as therapy against a solid, localized tumor system which does not directly affect the hematopoietic process.

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INTRODUCTION AND REVIEW OF THE LITERATURE

One of the major problems in cancer chemotherapy is the nonselective action of the antiproliferative agents used. The fact that nonmalignant as well as cancerous cells are affected results in severe toxicity problems, and therefore, chemotherapeutic agents in general cannot be used in dosages sufficient to destroy every neoplastic cell, a condition necessary for a true "cure" (Mihich, 1970).

Immunotherapy, on the other hand, has not been notably successful as a sole means of treatment of detectable malignancy, probably because the tumor cells, by this time, have achieved numbers far above that which antibody-forming cells can kill by themselves (Fass and Fefer, 1972). Nevertheless, there is the obvious advantage of the highly specific nature of the immune response (Klein, 1966) whereby antibodies can be made against new tumor-cell antigens. These antibodies would not react with normal cells that lack these antigens.

Recent approaches to this problem combine sublethal chemotherapy with immunotherapy (Fass and Fefer, 1972) in an attempt to compensate for the weaknesses of each system; adequately reducing tumor size by means of an appropriate chemical agent, then eradicating any remaining cells by existing immune mechanisms or by immunotherapy (Mihich, 1970).

Friend Virus Disease is a leukemia of the erythroid-myeloid series (Metcalf et al., 1959) induced by a virus

accidentally discovered by Charlotte Friend in the course of examining the Ehrlich ascites tumor (Friend, 1957). The virus itself is an ether-sensitive RNA virus which has been extensively described in electron-microscopic studies (deHarven and Friend, 1958, 1960a). The particle appears as two concentric shells, each a double membrane about 50 Å thick. The outer shell measures 87 mμ and the inner 52 mμ. The virus material is usually denser between the outer and inner membranes as compared to that in the central portion, giving the particle the doughnut shape characteristic of Type A virus particles. Such particles are frequently observed in a budding sequence, particularly in the megakaryocytes, both at the plasma membrane and in vacuoles within the cytoplasm. Particles also are seen in intimate contact with cell membranes of leukemic cells and budding from these membranes which form an envelope or peplos responsible for the ether sensitivity characteristic of the particle (Friend, 1957). Chan et al. (1968) similarly report virus particles in close association with cell membranes in the spleen.

In her original report, Friend (1957) also determined the physical stability of the viral agent, noting that filtrates of infected spleens were active after storage at -70°C for 6 months, at 4°C for 11 days, and when lyophilized for 3 months. Infectivity was destroyed by heating at 56°C for 30 minutes, by overnight exposure to ether at 4°C and by

treatment with a 1:200 dilution of formalin.

The disease itself centers in the hemopoietic tissue of the spleen and liver, causing marked splenomegaly and hepatomegaly, with death often resulting from splenic rupture. In addition, Friend (1957) described large mononuclear cells with rounded, lobed, or horseshoe-shaped nuclei, present in the peripheral blood. Metcalf et al. (1959) have termed these "Friend Cells" and include them with lymphocytes in classifying the hematology of the disease. Other pathological features include anemia, lymphocytosis, thrombocytopenia and many red blood cell abnormalities.

These same authors note that the first histological signs of the disease are observed in the spleen as early as four days after infection, when enlargement of reticulum cell foci is visible in the subcapsular area, along splenic trabeculae, and in the pulp spaces between lymphoid follicles. Numerous erythroblasts in various stages of development are noted around these foci and later, similar foci are observed in the liver.

Siegler and Rich (1965) propose that splenic enlargement is due to intense proliferation of erythrocyte precursors in the red pulp of the spleen, and that the failure of these cells to mature results in the anemia characteristic of the disease. This view is supported by tissue culture studies (Patuleia and Friend, 1967) showing that cultures of undifferentiated reticulum-cell sarcoma derived from

spleen or liver cells of Friend virus-infected animals eventually mature to proerythroblasts. The view of Boiron et al. (1965) that the so-called "Friend cell" is probably a proerythroblast further corroborates the erythroblastic nature of Friend disease, as does the work of Reilly and Schloss (1971) which proposed that erythrocytes of infected animals are carriers of the Friend virus particle.

Metcalf et al. (1959) note that a progressive lymphocytosis occurs the second day after infection and reaches levels of 15,000 - 30,000 / cu mm in 7 - 14 days. This, along with the findings of Ceglowski and Friedman (1969 and 1970) that the immunosuppressive effect of Friend virus-induced leukemia is based on interference with the maturation of lymphoid precursor cells suggests that the lymphoid cell line is involved in the disease.

In addition, the myeloid series is postulated as a possible target site for Friend virus replication in the work of Thomson (1969). He found that bone marrow, containing more myelocytes than lymphocytes, was better able to transmit Friend disease to lethally irradiated animals than was splenic tissue, containing more lymphocytes than granulocytes. Finally, megakaryocytes have been observed (deHarven and Friend, 1960b) as sites of viral budding, although these cells are not leukemic. Brodsky et al. (1968) propose, however, that this results in increased platelet fragility and consequent thrombocytopenia.

Much of the above evidence strongly suggests that Friend virus multiplies in pluripotential stem cells of the hemopoietic series. More recently, the work of Jacoby (1972) with Friend disease and leucogenenol proposes that the synergism between the virus and the molecule is attributable to the fact that leucogenenol stimulates proliferation of the very stem cells in which Friend virus replicates.

A final aspect of Friend disease to be considered is its immunosuppressive effect (Salaman and Wedderburn, 1966). In electron microscope studies, few normal, recognizable plasma cells or lymphocytes can be found in the spleens of Friend Virus-infected animals (Chan et al., 1968) and they propose that the immunologic defect caused by the virus may result from competition between antigens and virus particles for specific stem cells. This same view is reiterated by Ceglowski and Friedman (1970) who indicate a quantitative deficiency in the number of antibody-forming cells in spleens and livers of leukemic mice. Since once the immune mechanism has been stimulated by antigen, virus infection has only a slight effect, it seems logical that the immunosuppressive effect of Friend virus occurs in primitive, immunocompetent cells rather than at the level of the small, committed lymphocyte (Koo et al., 1971).

Such considerations directed the attention of Jacoby (1972) and this investigator to leucogenenol. This compound was found by Rice and Ciavarra (1971) to stimulate the

maturation rate of antibody-producing cells and consequently to elicit normal antibody titers in splenectomized animals.

Leucogenenol was first isolated by Rice (1966) from Penicillium gilmanii and later by Rice and Shaikh (1970) from normal bovine and human liver. Chemical analysis (Rice, 1971) indicated that the compound is an enol of the formula $C_{18}H_{25}NO_8$ and is 2-(1,2, dihydroxy-3-methyl-5-oxocyclohexyl)-3,11, dihydroxyl-11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro [5,5] undeca-2,4 dien-7-one. The molecule is of interest because of its ability to induce leucocytosis without a febrile response when injected into rabbits (Rice, 1966). In a later study, the same author (Rice, 1968) demonstrated that in mice the injection of leucogenenol stimulates marked lymphocytosis with a corresponding drop in neutrophils four hours after injection. Studies of the effect of leucogenenol on the cells of the bone marrow of mice (Rice and Darden, 1968) indicate an increase in myeloblasts after 24 hours followed by a return to normal levels at the end of 5 days. At the same time an apparent decrease in lymphocytes and lymphoblasts occurs. The transitory nature of this neutrophilia as well as the fact that neutrophil increase is followed by a decrease in promyeloblasts and myelocytes at the end of 48 hours indicates that leucogenenol acts by increasing the rate of production of one cell type from another, leading to the formation of the mature neutrophil. The lymphoid decrease is followed within

48 hours by a sharp increase in the number of lymphocytes (Rice, Lepick and Darden, 1968), indicating that leucogenenol stimulates the maturation and/or cell division of both myeloid and lymphoid cells. This conclusion is further supported by the finding that an increase in peripheral blood lymphocytes occurs in irradiated mice given leucogenenol and that spleen imprints from the same animals reveal a twofold increase in the relative number of lymphoblasts 24, 48 and 120 hours after injection.

Further studies on irradiated mice (Rice, Lepick and Hepner, 1970) demonstrate more rapidly arising and increased hemolysin titers in animals treated with leucogenenol as compared to those untreated. Since lymphoid cells are known producers of antibody it is again suggested that the compound increases the rate at which these cells recover from the effects of X-radiation damage. A more recent study (Rice et al., 1972) notes that injection of leucogenenol together with sheep erythrocytes into splenectomized rats results in the formation of normal titers of 19S (IgM) hemolysin. The authors suggest that leucogenenol acts by increasing the rate of transformation of immunoincompetent cells into antigen-reactive lymphocytes.

Autoradiographic studies of the action of leucogenenol on the blood cells of the rat (Rice, McCurdy and Aziz, 1971) whereby tritiated thymidine was injected concurrently with leucogenenol, demonstrate that the most intense label is

found in the proliferating pool of cells in the bone marrow and is then transferred to cells in the myeloid series. Likewise, spleen imprints show a significant increase in the percentage of labeled lymphocytes following leucogenenol injection. In the same study, it is suggested that although leucogenenol does not appear to have any striking action of erythroid cells, the significant increase in labeled pro-rubricytes 12 and 24 hours after administration of leucogenenol may suggest that the compound stimulates the maturation of at least one type of nucleated erythroid cell.

More extensive autoradiographic studies in rats (Rice, Connolly, Aziz and McCurdy, 1971) attribute the decrease in peripheral neutrophils 12 hours after injection to an efficient sequestering mechanism in the animal, a proposal supported by the concomitant increase in labeled neutrophils in the spleen. The same study points out that the lymphocytosis occurring after injection of leucogenenol is accompanied by an even greater increase in labeled lymphocytes, indicating increased maturation of lymphoid precursors, a finding which is in agreement with the previously noted suggestion that leucogenenol increases the rate of transformation of antibody-producing cells (Rice et al., 1972).

Finally, studies on the action of leucogenenol on lymphoblastoid cells of normal and neoplastic origin propose that leucogenenol somehow affects the enzyme systems that are associated with the transformation of cells (Rice and McCurdy,

1971). Further, Rice, Blum and Rene (1970) suggest that the fact that leucogenenol is a normal constituent of liver tissue implies that it may play a role in the normal growth and regulation of hemopoietic cells.

It is thus strongly suggested that leucogenenol is instrumental in regulating the proliferation and transformation of stem cells, possibly the same cells in which Friend virus is presumed to replicate. These findings were supportive of those of Jacoby (1972) who, working with the interaction of Friend virus disease and leucogenenol, found that the compound potentiates the leukemia, probably by providing more sites in which the virus can replicate; more cells which can be transformed by it. At the same time, however, leucogenenol evidently does counteract the immunosuppressive effect of Friend disease, as evidenced by the production of near normal antibody titers in infected animals treated with leucogenenol. Nevertheless, it is proposed that these normal antibody levels are inefficient against massive levels of both viral particles and tumor cells.

It is possible that the beneficial aspects of leucogenenol might be better exploited if an appropriate course of chemotherapy were first administered in an attempt to reduce the number of neoplastic cells. Thus, the folic acid antagonist Methotrexate was chosen as an antiproliferative agent in these experiments.

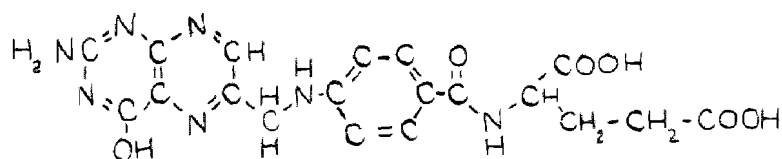
The basis for an understanding of the action of

Methotrexate lies in the role of folic acid (pterylglutamic acid), a compound isolated in the 1940's and associated with hemopoietic activity as evidenced by its therapeutic effect on some anemias (Pfiffner et al., 1947). The experimental use of folic acid antagonists demonstrated a competition between the antagonist and folic acid resulting in a general slowing of growth, reduction in the percentage of hemoglobin and a reduced leucocyte count (Franklin et al., 1947). This work suggested that since folic acid antagonists evidently depress cytopoietic mechanisms they may be useful in treating diseases of erythrocytosis or leucocytosis.

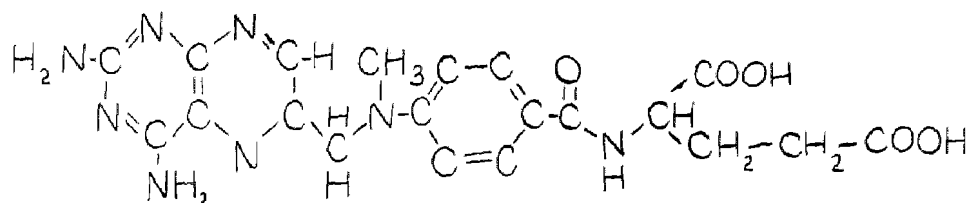
Farber et al. (1948) rather successfully employed aminopterin, a closely related predecessor of Methotrexate (amethopterin) in the treatment of leukemia in children. The synthesis of a methylated analogue of aminopterin followed that of aminopterin and its effects on experimental animals were studied (Seeger et al., 1947). Inhibition of growth, anemia, leucopenia and granulocytopenia were noted by Franklin et al. (1949), who also suggested that the compound might be used as a chemotherapeutic agent in the treatment of myelogenous leukemia. Such experiments were conducted and Methotrexate was shown to prolong the survival time of mice infected with leukemia AK4 (Burchenal et al., 1949). The observation that reduced folic acid compounds blocked the effect of Methotrexate led to the suggestion that its mode of action involved blocking the biochemical reduction of

folic acid to tetrahydrofolic acid (Jukes, 1953). In work with leukemic mice, Balis and Dancis (1955) note that thymidilic acid biosynthesis is the reaction most sensitive to the action of folic acid antagonists.

The enzyme system needed for the reduction of folic acid was obtained in partially purified form from chicken livers by Futterman (1957), who also demonstrated that aminopterin blocked the action of the reductase. In the following year amethopterin (Methotrexate) was shown to have the same blocking effect (Osborn et al., 1958). These authors state that the primary site of action is probably only at the step which reduces dihydrofolic acid to tetrahydrofolic acid. Peters and Greenberg (1959) found that the enzyme, as extracted from sheep liver, could be titrated with aminopterin, a finding which is explained by the fact that the binding of the drug to the enzyme is so strong that the dissociation constant is quite small (Werkheiser, 1961). The close similarity between the structure of folic acid and that of Methotrexate further supports this mechanism. Folic acid:



Methotrexate:



Holland (1961) notes that the inactivation of folic reductase by folic antagonists disrupts the capacity for cell multiplication by interfering with nucleic acid biosynthesis, thereby producing the clinical signs of leucopenia, thrombocytopenia and erythrocytopenia; symptoms associated with tissues noted for fairly rapid proliferation. Delmonte and Jukes (1962) propose that the effects of Methotrexate are due to the blocking of the enzyme dihydrofolic reductase, and thereby interfering with the production of tetrahydrofolic acid, a compound which, after several intermediate reactions, transfers carbon atoms to deoxyuridilic acid to form thymidilic acid, a component of DNA (Jukes, 1961). Werkheiser (1963) also notes that a lesser inhibition of purine synthesis has been found. Thus, the lethal action of Methotrexate is likely to be on cells that are in rapid DNA synthesis (Blackett, 1968) and more specifically on those in the S-phase of the generation cycle (Bruce et al., 1966). Borsa and Whitmore (1969) further note that although cells outside the S-phase are not killed, their progression through the cell cycle may be prevented by the inhibition of purine

synthesis. The same study points out that if purines are added to the culture medium, thereby inhibiting thymidilic acid synthesis only, cells in the G, M, and G₂ phase lose their viability when they reach the S phase.

Thus, it is rather surprising that Methotrexate has not been notably successful in the therapy of Friend disease. Mirand et al. (1961) observed that the drug caused a significant decrease in spleen size and decreases in Fe⁵⁹ uptake, by the spleen but no increase in survival time. Along similar lines, Chirigos et al. (1965) report the same decreases in spleen size induced by methotrexate, but no decrease in virus recovery from infected animals.

Methotrexate also acts as an immunosuppressive agent (Uphoff, 1958). In studies with guinea pigs it was shown that animals sensitized with Bacille Calmette-Guerin (BCG) then given Methotrexate, failed to develop positive skin tests, although viable lymph node cells from these animals were able to transfer tuberculin hypersensitivity (Friedman and Buckler, 1963). If animals were treated with Methotrexate after being given such sensitized cells, hypersensitivity failed to develop until the drug was discontinued. The conclusion was reached that Methotrexate selectively inhibited the proliferation of immunologically active cells. This conclusion is further supported by a reversal in differential white cell counts (Friedman, 1964). In Methotrexate-treated guinea pigs, the counts were 66% polymorphonuclear neutrophils

(PMN's) and 34% lymphocytes, whereas in untreated animals the proportion was 38% PMN's and 62% lymphocytes. Both groups had also received BCG.

Thus, some problems arise regarding the use of Methotrexate in Friend disease therapy. An important question is why the drug seemingly does not prolong the survival time of infected animals. Mirand et al. (1961) suggests that the parameters of spleen weight and Fe^{59} uptake are more sensitive indicators of drug effect than mortality, and Chirigos et al. (1965) proposes that the intensity of viremia was simply too great for the drug to be effective. Another possibility might be the toxicity of the compound, which could result in the early death of the animals. Further, the immunosuppressive effect of Methotrexate may also be an important factor in the failure of the drug to prolong survival time.

Bacille-Calmette Guerin (BCG) is an attenuated strain of Mycobacterium bovis accidentally discovered in 1906 by Calmette and Guerin as they were culturing the virulent parent strain on glycerine-bile-potato medium. (Calmette and Guerin, 1906). During the 1920's BCG was used prophylactically in young infants in the United States, although considerable controversy surrounded its use. Today, BCG is used extensively throughout the world except in the United States as a means of immunization against tuberculosis.

The observation that survivors of tuberculosis were

less susceptible to neoplastic growth led several investigators to the experimental use of BCG therapy in conjunction with malignant growths. Old et al. (1959) injected BCG into mice, then inoculated the animals with several types of transplantable tumors. The tumors grew normally for the first 8 to 10 days, then 70 - 75% of them regressed. An increase in spleen weight accompanied by multiple large granulomas implicates reticulo-endothelial involvement as a possible mechanism (Old et al., 1961). Weiss et al. (1961) found both living BCG as well as phenol-killed, acetone-extracted and methanol-extracted organisms to be effective when given before tumor transplants. Retardation in onset of tumor development, prevention of tumor development, slowing of tumor growth, regression of tumor, inhibition of metastasis and increase in life span were some notable effects.

Many varying hypotheses have been advanced to explain the mechanism of action of BCG, but very few conclusions have been reached. Lemonde and Clode (1962) suggest that increased phagocytic activity and an increased antibody response are responsible for increasing survival time in leukemic mice. They also postulate that the mild inflammatory disturbance caused by BCG infection may increase the secretion of hormones such as the corticosteroids which may inhibit neoplasms. Studies with skin graft rejection led to the observation that BCG infection results in a greater number of functionally altered phagocytic cells and exerts a proliferative

stimulus on cells of the reticuloendothelial system, which in turn may cause more vigorous production of immunocompetent cells (Old et al., 1961).

Skin graft studies were also employed by Vitale and Allegretti (1963) who also implicate the reticuloendothelial system in BCG-induced reactions. It also has been noted that BCG infection in mice increases the total number of nucleated spleen cells as well as the number of plaque-forming cells as tested with sheep erythrocytes (Stjernsward, 1966). He further suggests that since BCG can increase the number of antibody-producing cells to an unrelated antigen, its effect might be due to nonspecific proliferation of stem cells. Another possibility is that increased phagocytosis and therefore increased uptake of antigen may lead to more immunologically competent cells. Lemonde and Clode (1966) propose that protection results not from antibodies directed against the virus but possibly from those against antigens induced in cells made neoplastic by the virus, i.e., tumor antigens. This view is supported by studies involving Adenovirus type 12 inoculated into mice which were given BCG 4 weeks later (Sjogren and Ankerst, 1969). These authors propose that BCG immunity involves nonspecific transplantation antigens, since BCG was given at a relatively late stage of the latent period when an antiviral immune response would no longer be possible. A study of cellular kinetics by North (1969) shows that BCG infection resulted in intense

proliferation of lymphoid cells in the spleen and proliferation of macrophages in the peritoneal cavity.

Zbar et al. (1970) inoculated tumor cells mixed with BCG into guinea pigs and noted an inflammatory response to BCG but no tumor growth. The animals were immune to further challenge by tumor cells of the same type. A delayed cutaneous hypersensitivity to the tumor cells was noted. Continued work with guinea pigs points to a possible mechanism of delayed hypersensitivity (Zbar and Tanaka, 1971). Further, actual contact between BCG and tumor cells is proposed as necessary for participation of the host's immune defenses (Zbar et al., 1971). Such participation is deemed essential since guinea pigs with impaired defenses are unable to suppress tumor growth even in the presence of BCG.

Considerable confusion arises in the literature of the past few years regarding the nature of the response to BCG. Schwartz et al. (1971) suggest that BCG initiates a local immunological response which alters the oncogenicity of the virus in the area, but state that the mechanism for this is not known. Along the same line of thought, Lemonde et al. (1971) found that in mice and hamsters given BCG, antibody titers to polyoma virus increased, suggesting that BCG stimulates tumor immunity, evidently of a humoral nature. This position is contradicted by Reif and Kim (1971) who found that BCG did not stimulate primary antibody responses to sheep erythrocytes and suggest that cellular rather than

humoral responses are involved. Likewise, the finding that sera from patients with growing tumors could block the cytotoxic effect of lymphocytes immune to those tumors, implies that cellular rather than humoral responses are of benefit in tumor immunity (Hellstrom et al., 1971). Further support is offered by Sokal et al. (1972) who, upon injection of BCG and cultured cells into leukemia patients stimulated delayed hypersensitivity to the antigens of the target cells with little humoral antibody production.

Further debate arises regarding the specific or non-specific nature of the anti-tumor response induced by BCG. Working with mice, Bartlett et al. (1972) suggest that host immune reactivity to BCG was necessary and possibly even sufficient to prevent tumor growth at the site of BCG injection. Thus, tumor-specific immunity is not considered necessary, but the tumor cells and the BCG must be in the same locale. The suggestion is made that tumor destruction may be due to nonspecific effects of specific reactivity to BCG. A possible mechanism in conjunction with this is suggested by Williams and Granger (1969) who propose that lymphocytes activated by antigen exposure may be nonspecifically cytotoxic. They note that cell destruction is initiated by lymphotoxins, perhaps in a nonspecific manner. Somewhat in contrast to this Pearson et al. (1972) suggest that BCG dramatically enhances a tumor-specific response in addition to activating nonspecific elements of the reticuloendothelial

system.

More recent studies describe a granulomatous histiocytic reaction at the tumor site and in the draining lymph nodes after BCG infection (Hanna et al., 1972). These authors raise the question of whether this reaction might be classified with delayed hypersensitivity in the realm of cellular immunity, pointing out that while granulomatous inflammation is often a major characteristic of antimicrobial cellular immunity, histiocytes do accumulate in delayed-type antitissue reactions. It is suggested that the histiocytes may be necessary for the more predominant lymphocytic reaction in antitissue immunity, and that lymphocyte sensitization may occur during the granulomatous reaction. Thus, there are many speculations and few conclusions regarding the mechanism of BCG action on tumors, save a tentative one that the process of tumor destruction is immunologically nonspecific.

Finally, BCG has been shown to be effective therapy against Friend disease. Larson et al. (1970) administered BCG before Friend virus and noted that the mortality rate among treated animals was significantly less. In a later study (Larson et al., 1972) BCG was given 1 or 3 weeks after Friend virus. Spleen weights were significantly lower, fewer foci were present in the spleens, and life spans were lengthened in BCG-treated mice.

This study represents an attempt to retard the progression of Friend virus disease in BALB/c mice, employing

Methotrexate chemotherapy as a means of reducing the population of tumor cells, and leucogenenol and BCG immunotherapy as a means of stimulating the animals' own immune responses to the tumor cells.

METHODS AND MATERIALS

Mice

Young adult female BALB/c mice, weighing about 20 g were obtained from Simonsen Laboratories, Gilroy, Calif. and from Cumberland View Farms, Clinton, Tenn. They were kept in disposable plastic cages in groups of no more than 10. Sterilized sawdust bedding was used. The mice were given water and standard laboratory chow ad libitum.

Friend Virus

The Friend Virus was obtained from the stock pool maintained in our laboratory. The history of this stock has been described by Elliott et al. (1970) and by Elliott and Schloss (1971). The ID_{50} was $10^4/0.2$ ml given intraperitoneally. A 10^{-3} dilution was made as follows: After removal from $-70^{\circ}C$ storage, the virus stock was thawed by gentle swirling in a $37^{\circ}C$ water bath. About 2 ml of the suspension were transferred to a sterile centrifuge tube and spun for 10 minutes at 2000 XG. A 10^{-1} dilution, then a 10^{-3} dilution of the supernatant were made using millipore-filtered sucrose stabilizer (Elliott et al., 1970). Dilutions

were made at a temperature close to 0°C maintained by ice. In all experiments, 0.2 ml of 10^{-3} virus dilution were injected intraperitoneally.

Leucogenenol

Leucogenenol was isolated from lyophilized bovine liver (Nutritional Biochemicals Corporation, Cleveland, Ohio) according to the procedure of Rice and Shaikh (1970) as modified by Jacoby (1972). In all experiments, 0.4 μg dissolved in 0.2 ml sterile, pyrogen-free water were injected intraperitoneally.

Methotrexate

Methotrexate was obtained in powdered form from Nutritional Biochemicals Corporation, Cleveland, Ohio. This was dissolved in a sterile 4% solution of NaHCO_3 prepared in pyrogen-free water and given in a dose of 2.5 mg/kg subcutaneously (Floersheim, 1970).

BCG

Bacille Calmette-Guerine (BCG) was obtained as a frozen suspension from Trudeau Institute, Inc., Saranac Lake, New York, through the courtesy of Dr. G. P. Kubica. It was kept at -70°C until use. This suspension was diluted with .15M sterile saline and administered intraperitoneally in a dose of 4.0×10^6 viable units in 0.2 ml.

Experimental Design

A total of 67 mice were divided into 8 groups. The groups varied in size because certain animals had been infected with parasites and had to be eliminated from the experiment. The groups were treated as follows:

- Group 1: Untreated Control group. 8 mice.
- Group 2: Control group; injected with Friend Virus only. 8 mice.
- Group 3: Inoculated with Friend Virus. Leucogenenol administered 12 and 19 days after infection. 9 mice.
- Group 4: Inoculated with Friend Virus. BCG administered 14 days after infection. 9 mice.
- Group 5: Inoculated with Friend Virus. Methotrexate given on three consecutive days beginning 7 days after infection. 8 mice.
- Group 6: Inoculated with Friend Virus. Methotrexate given on three consecutive days beginning 7 days after infection. Leucogenenol given 12 and 19 days after infection. 11 mice.
- Group 7: Inoculated with Friend Virus. Methotrexate given on three consecutive days beginning

7 days after infection. BCG administered
14 days after infection. 9 mice.

Group 8: Inoculated with Friend Virus. Methotrexate given on three consecutive days beginning 7 days after infection. Leucogenenol given 12 and 19 days after infection. BCG administered 14 days after infection. 5 mice.

The animals were checked several times each day to remove dead mice. The dates of the animals' deaths were recorded. At death, each mouse was weighed and autopsied. The spleen and liver were excised and also weighed, and the ratios of the weights computed.

Differential blood counts were made 2, 4, and 6 weeks after infection. Peripheral blood samples were obtained from tail snips and the smears were stained with Wright's stain and examined at 1000X. A total of 100 cells were counted and classified. Questionable cells were examined by several qualified people.

Statistical analysis was conducted by a standard t-test, comparing various animal groups. The p values were then determined from a standard table. In general, significance was ascribed to a p value of < 0.10 . In several instances, values extremely close to 0.10 are reported as > 0.10 .

RESULTS

In Table 1 and Figure 1, there was no difference in survival time between FV-infected animals which were treated with either leucogenenol or BCG and those that were left untreated (p not significant). Infected animals treated solely with Methotrexate (MTX) did show a significant increase ($p < .05$) in lifespan over those infected but not treated. Animals treated with both MTX and leucogenenol also had a substantially longer lifespan ($p > .1$) than untreated animals. The same was true for animals treated with MTX and BCG ($p > .1$). Treatment with MTX, leucogenenol and BCG significantly prolonged the life of infected animals as compared to infected but untreated controls to approximately the same extent as did treatment with MTX alone.

Treatment with MTX and leucogenenol resulted in a significantly longer lifespan ($p < .1$) than did treatment with leucogenenol only. Treatment with both MTX and BCG resulted in a slightly but nonsignificantly longer lifespan than did treatment with BCG alone. No significant differences in survival time were noted when leucogenenol was added to MTX treatment, when BCG was added to MTX treatment or when both leucogenenol and BCG were added to MTX treatment. Also, no significant difference was found when BCG was added to treatment with MTX and leucogenenol, nor when MTX and leucogenenol were added to treatment with BCG. Nor

Table 1. Mean survival time in days of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG.

GROUP	MEAN SURVIVAL TIME (days)	RANGE (days)
1 No treatment	Terminated - 146	----- (8)*
2 FV only	46.63	40-62 (8)
3 FV + Leucogenenol	43.70	39-47 (9)
4 FV + BCG	46.50	35-61 (9)
5 FV + MTX	56.25	39-72 (8)
6 FV + MTX + Leucogenenol	57.27	38-96 (11)
7 FV + MTX + BCG	51.78	43-55 (9)
8 FV + MTX + BCG + Leucogenenol	55.00	53-59 (5)

* Number in parentheses represents the number of mice used in calculating each value.

Figure 1. Mean survival time in days of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG. Untreated controls not included.

Group 2: FV only

Group 3: FV + Leucogenenol

Group 4: FV + BCG

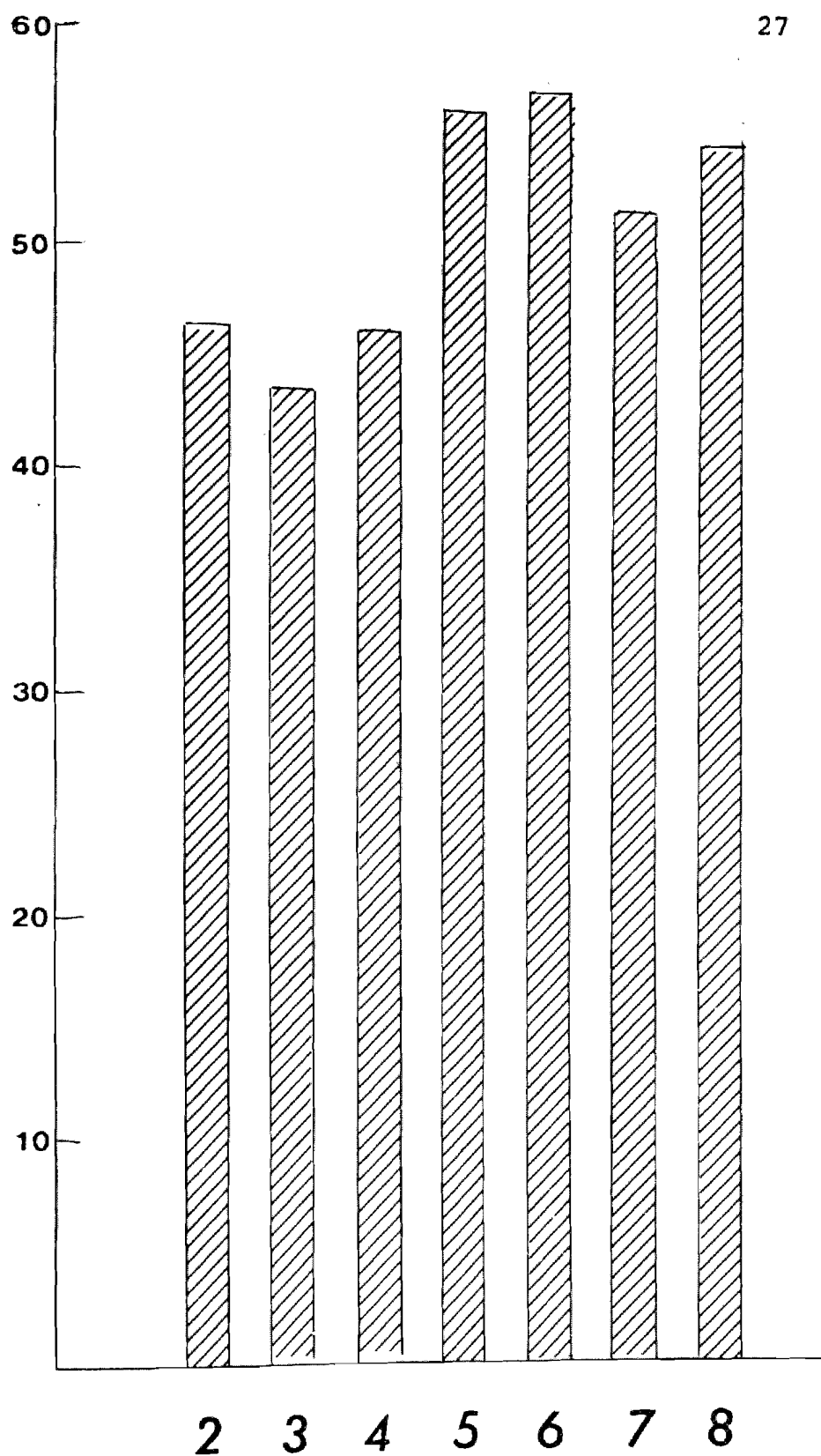
Group 5: FV + MTX

Group 6: FV + MTX + Leucogenenol

Group 7: FV + MTX + BCG

Group 8: FV + MTX + BCG + Leucogenenol

SURVIVAL TIME IN DAYS



ANIMAL GROUPS

was a significant difference observed between animals treated with MTX and BCG and those treated with MTX, BCG, and leucogenenol.

The greatest significant difference in lifespan ($p < .001$) was observed between infected animals given only leucogenenol and those infected mice treated with MTX, leucogenenol and BCG, the latter having the longer survival time.

No significant differences were observed in spleen and liver/total body weight ratios in any of the groups except that the ratio for all FV-infected animals, no matter what subsequent treatment, if any, was given, was much greater than that of normal, noninfected mice ($p < .001$). These data can be found in Table 2 and Figure 2.

Table 3 and Figure 3 show the data comparing the percentages of lymphocytes among the various treatment groups at different times in the experiment. Treatment with leucogenenol as compared with lack of such treatment in the control animals significantly raised the percentage of lymphocytes in week 2 ($p < .025$) but not in week 4 or 6 (p not significant). In contrast, treatment with BCG alone did not result in any significant difference in lymphocytes between this group and untreated controls. As compared to untreated but infected controls, animals treated with MTX alone showed a significant drop in the number of lymphocytes at weeks 2 ($p < .005$), 4 ($p < .005$) and 6 ($p < .05$) as did animals treated with MTX and leucogenenol ($p < .005$, $p < .05$

Table 2. Average ratios of spleen and liver weight to total body weight of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG.

GROUP	AVERAGE SPLEEN + LIVER WT. TOTAL BODY WEIGHT	RANGE
1 No treatment	0.063	.057-.071 (8)*
2 FV only	0.287	.248-.320 (5)
3 FV + Leucogenenol	0.302	.280-.340 (6)
4 FV + BCG	0.315	.243-.415 (5)
5 FV + MTX	0.295	.217-.354 (7)
6 FV + MTX + Leucogenenol	0.306	.249-.387 (8)
7 FV + MTX + BCG	0.325	.255-.367 (8)
8 FV + MTX + BCG + Leucogenenol	0.307	.276-.333 (5)

* Number in parentheses represents the number of animals used in computing ratios. Some animals could not be used because of ruptured spleens.

Figure 2. Average ratios of spleen and liver weight to total body weight of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG.

- Group 1: Untreated controls
- Group 2: FV only
- Group 3: FV + Leucogenenol
- Group 4: FV + BCG
- Group 5: FV + MTX
- Group 6: FV + MTX + Leucogenenol
- Group 7: FV + MTX + BCG
- Group 8: FV + MTX + BCG + Leucogenenol

WEIGHT RATIO = $\frac{\text{SPLEEN} + \text{LIVER WT.}}{\text{TOTAL BODY WT.}}$

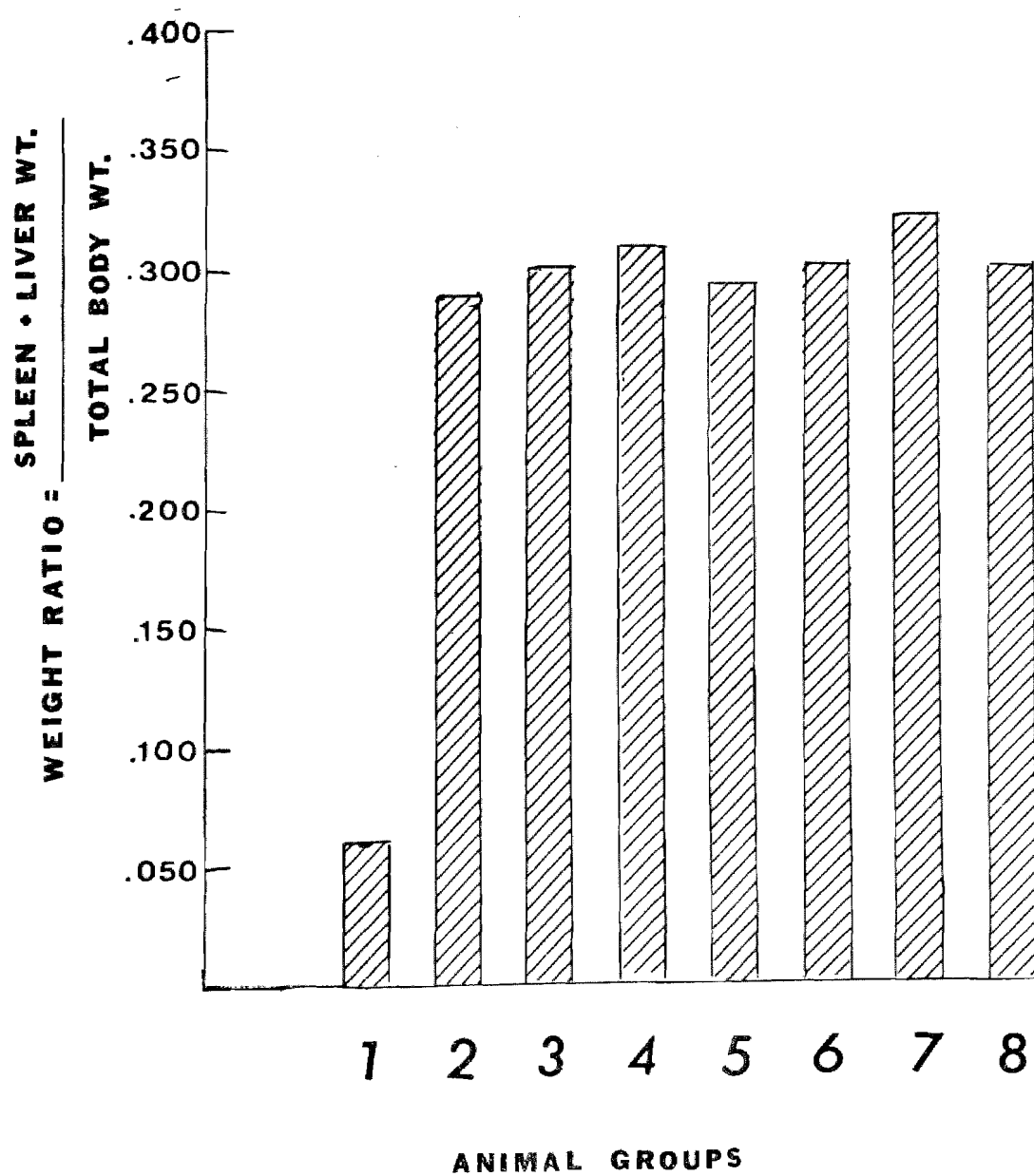


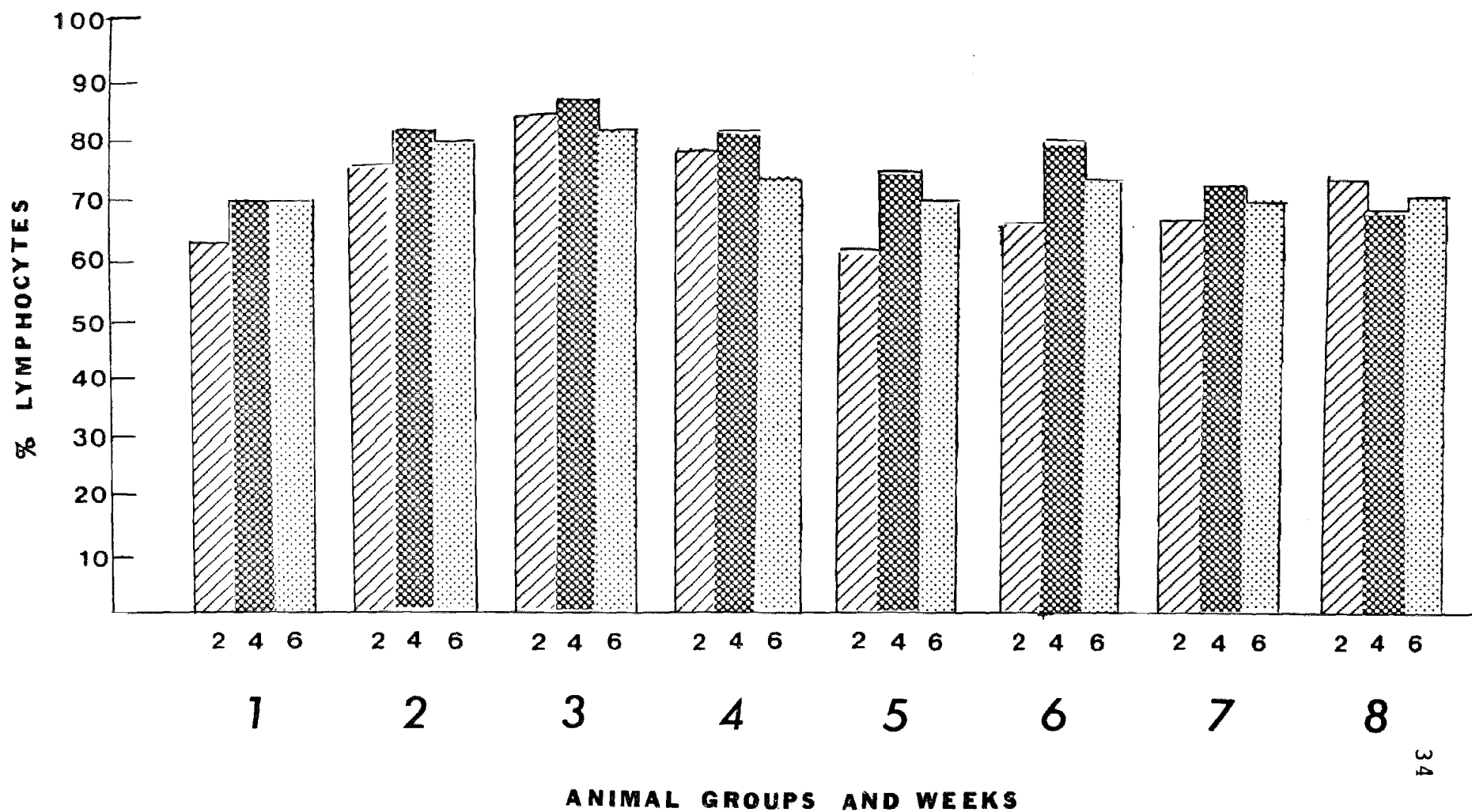
Table 3. Average percentage of lymphocytes in peripheral blood smears of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG.

GROUP	WEEK 2 % LYMPHS	WEEK 4 % LYMPHS	WEEK 6 % LYMPHS
1 No treatment	65.6 (9)	71.7 (8)	71.3 (8)*
2 FV only	77.5 (8)	83.8 (8)	80.0 (5)
3 FV + Leucogenenol	84.4 (9)	87.3 (9)	82.0 (4)
4 FV + BCG	78.1 (9)	83.4 (5)	73.3 (6)
5 FV + MTX	63.3 (8)	75.1 (8)	71.3 (7)
6 FV + MTX + Leucogenenol	67.3 (11)	79.8 (11)	73.5 (10)
7 FV + MTX + BCG	67.7 (9)	72.7 (9)	69.6 (9)
8 FV + MTX + BCG + Leucogenenol	75.7 (5)	68.2 (5)	71.6 (5)

* Number in parentheses represents the number of slides used in computing the mean.

Figure 3. Average percentage of lymphocytes in peripheral blood smears of BALB/c mice injected with various combinations of FV, leucogenenol, Methotrexate (MTX) and BCG.

- Group 1: Untreated controls
- Group 2: FV only
- Group 3: FV + Leucogenenol
- Group 4: FV + BCG
- Group 5: FV + MTX
- Group 6: FV + MTX + Leucogenenol
- Group 7: FV + MTX + BCG
- Group 8: FV + MTX + BCG + Leucogenenol



and $p < .10$). Significant decreases in the percentage of lymphocytes were also found in animals treated with MTX and leucogenenol as compared with those treated with leucogenenol alone in weeks 2 ($p < .001$) and 4 ($p < .005$). However, a significant rise in the percentage of lymphocytes occurred in week 4 ($p < .05$) and slight but nonsignificant increases took place in weeks 2 and 6 when leucogenenol was added to MTX therapy.

Animals that received MTX and BCG showed a significant drop in the percentage of lymphocytes over those not treated at all sampling times ($p < .005$, $p < .005$ and $p < .01$). Adding BCG to treatment with MTX, however, produced no significant differences in the percentage of lymphocytes, but adding BCG to treatment with MTX and leucogenenol produced a significant drop in week 4 ($p < .001$). Also, adding BCG and leucogenenol to treatment with MTX produced a drop in the percentage of lymphocytes in week 4 ($p < .05$). There was no significant difference in week 6. Animals treated with MTX and BCG showed a significant drop in the percentage of lymphocytes in weeks 2 ($p < .01$) and 4 ($p < .005$) when compared to mice treated with BCG only, but adding both leucogenenol and MTX to BCG treatment produced a significant drop only in week 4 ($p < .005$). Mice treated with MTX and BCG in addition to leucogenenol showed significant drops in the percentage of lymphocytes as compared with animals given only leucogenenol in weeks 2 and 4 ($p < .001$ and $p < .1$). Adding

leucogenenol to MTX and BCG treatment produced a significant rise in the percentage of lymphocytes in week 2 ($p < .1$) but no significant differences in weeks 4 and 6.

DISCUSSION

Methotrexate was significantly effective in prolonging survival time of FV infected mice. This is in marked contrast with the report of Mirand et al. (1961) and may be accounted for by several factors. Mirand used HA/ICR Swiss mice rather than the BALB/c animals used here, and he administered MTX in a different dosage and time schedule, employing smaller doses of 1 mg/kg over a longer period of 11 days, beginning 24 hours after FV infection. In this experiment, a dose of 2.5 mg/kg was given for 3 consecutive days beginning 7 days after infection. Also, a different strain of FV was employed. Mirand (1966) uses a polycythemic strain whereas the strain used in these experiments is anemia-producing. All of these differences are important factors in judging the effectiveness of MTX treatment of Friend Virus Disease.

The beneficial effect of MTX in these experiments is not surprising from a biochemical point of view. Since the compound prevents the formation of thymidilic acid by competing with folic acid for the reductase (Delmonte and Jukes, 1962), it should be expected to be detrimental to any rapidly dividing cells, including those of Friend Virus Disease.

Further, since Mirand et al. (1961) report that the compound did have some effect on certain parameters of the disease, such as spleen weight and Fe^{59} uptake, the results reported here simply seem to extend those findings.

In all cases in these experiments, animals treated with MTX had significantly longer lifespans than those not so treated. Since the known molecular action of the drug is the blockage of DNA synthesis by preventing thymine formation (Delmonte and Jukes, 1962), it can be said that the drug's main effect is probably due to the destruction of the rapidly proliferating tumor cells. MTX also acts to prevent the development of T-lymphocytes which are active in cell-mediated immune responses to neoplastic cells (Friedman and Buckler, 1963). Thus it is noted that all animals that received MTX treatment in these experiments exhibited significantly lower percentages of lymphocytes in peripheral blood. Since the suppression of T-lymphocytes is extremely detrimental in the treatment of leukemia, it seems that the beneficial effect of MTX in destroying tumor cells outweighs the destruction of T-lymphocytes, at least under the conditions described for these experiments.

BCG alone had no effect on either survival time or on the percentage of lymphocytes in the peripheral blood. This is in direct contradiction to results obtained by Larson et al. (1970 and 1972). In his first experiments with BCG and FV, the author immunized mice with BCG before infecting them

with FV. This seems to be an artificial situation since a disease cannot be treated before it is present. In the later experiments, BCG was given either 1 or 3 weeks after infection by the intravenous route. The size of the BCG inoculum was the same as that used here, but further comparisons are impossible since Larson does not mention the strain of mice or BCG used nor does he describe the history of the FV pool employed. The results reported here are, perhaps, more in agreement with the work of Hanna, Zbar and Rapp (1972) in which tumor regression was noted when BCG was injected directly into the tumor site. They explained the beneficial results of BCG therapy as consisting primarily of a localized granulomatous inflammatory reaction. Since Friend Virus Disease is not localized but systemic and infiltrates a great variety of tissues, it is not so surprising that BCG showed little effect since a granulomatous inflammation simply cannot occur in all the organs and areas which FV infiltrates.

Further, adding BCG to MTX therapy had no appreciable beneficial effect. It is postulated that the T-lymphocytes were so suppressed by the drug that BCG stimulation was ineffective in restoring their ability to develop reactivity, thus decreasing the destructive effect that these cells would have upon any tumor cells present. Likewise, treatment with leucogenenol, another immunostimulant, had no effect after the administration of MTX. Perhaps here too,

MTX so efficiently suppressed immunocompetent cells that stimulation was impossible. Also, it is known that leucogenol stimulates the production of humoral antibodies (B-cells) but it is not yet known what effect, if any, the drug has on T-lymphocytes. If the compound does, in fact, stimulate only B-cells, no beneficial results could be expected since tumor rejection is mediated by cell-bound antibodies produced by T-cells and blocked by humoral antibodies produced by B-cells. As with BCG, however, the possibility exists that MTX suppressed both tumor cells and T-lymphocytes to the extent that stimulation by leucogenol was simply not enough to restore the production of the cytotoxic anti-tumor T-lymphocytes. Along the same lines, these present experiments suggest that even immunostimulation with both BCG and leucogenol was not sufficient to restimulate the T-cells suppressed by MTX.

The effects of MTX, however, were not permanent and after treatment was discontinued some evidence of "overshoot" (Pfeiffer and Tolmack, 1967) was present as increased but non-significant spleen-liver ratios. Even though MTX was effective in reducing tumor cell population in the beginning, as the drug was eliminated and newly synthesized folic acid reductase was not being blocked by the MTX, the remaining cells were again able to proliferate rapidly. Also, at this time there was less hindrance from T-lymphocytes since their development had been suppressed by the drug and

a longer period of time is required for their maturation, as well as for the development of the lymphotoxic effect of these T-cells. Thus, the progression of the disease was temporarily slowed by MTX treatment as shown by increased survival time, but the spleen-liver ratios did not show this effect since measurements were taken at the time of death from leukemia rather than at a time of maximum drug inhibition. In essence, when animals are given even effective drug treatment but the treatment is then stopped, the progression of the disease continues in the same manner as it would without treatment, although lifespan is increased. This is further supported by the data showing that in almost every case the beneficial effect of chemotherapy and immunotherapy on the percentage of lymphocytes in the peripheral blood had disappeared by the sixth week.

Although the slight drop in survival time as well as the elevation of spleen-liver ratios produced by leucogenenol were not significantly different from those of FV-infected but untreated animals, there was a definite and significant increase in the percentage of peripheral lymphocytes indicating that the drug did have an effect on these cells. Since it is known that leucogenenol stimulates the proliferation of cells producing humoral antibodies, or B-lymphocytes (Rice, Lepick and Hepner, 1970) it can be postulated that leucogenenol simply may provide more replication sites for the virus. This data supports the conclusions of Jacoby (1972) and also

explains why the addition of leucogenenol to MTX treatment had no beneficial effect in these experiments. The immunosuppressive effect of FV due to virus invasion of B-cells cannot be overcome by treatment with leucogenenol, even though the drug does stimulate antibody production in non-infected animals, since the cells resulting from leucogenenol stimulation will be transformed by the virus and the whole FV leukemic process proceeds more rapidly in the presence of elevated numbers of viruses (Fieldsteel et al., 1961).

It would be of value to repeat these experiments with several important modifications. First, some animals should be terminated after administering a complete course of drug and immunotherapy and spleen-liver ratios determined at that time should be compared with those of untreated animals. It would be expected that these weights would be significantly lower than those of untreated animals. Secondly, a second or even third course of drug and immunotherapy should be given after the animals had a sufficient time to allow for recovery from any toxic drug effects. In this way, newly proliferating tumor cells would be destroyed several times and the antibody producing ability of these animals would have been enhanced several times. With multiple drug and immunotherapy there perhaps would be a statistically significant increase in life span as compared to animals treated only once.

Furthermore, leucogenenol needs to be tested further in a localized tumor system which does not directly involve

the hemopoietic system. It is suggested that perhaps in such a system stimulation of elevated antibody titers would have a significantly beneficial effect in the destruction of tumor cells.

SUMMARY

These experiments were designed to investigate whether the immunostimulants leucogenenol and BCG when added to Methotrexate chemotherapy could retard the progression of Friend Virus Disease. Although Methotrexate therapy did prolong survival time, cessation of treatment was followed by evidence of normal progression of the leukemia and thus, at death, there were no significant differences in spleen-liver weight ratios when treated animals were compared to untreated controls. The data also indicate that MTX decreased the percentage of lymphocytes in the peripheral blood during the course of its administration. Treatment with immunostimulants had no appreciable effect on the progress of the disease. It is therefore concluded that the effect of Methotrexate therapy was to suppress the production and sensitization of T-lymphocytes so efficiently that consequent attempts at immunological restimulation were ineffective. Furthermore, this suppression was sufficiently great so that upon cessation of Methotrexate therapy the proliferation of leukemic cells exceeded that of new immunocompetent T-lymphocytes.

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